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REPRODUCTIVE BIOLOGY OF *ISOTRIA MEDEOLOIDES* (ORCHIDACEAE)

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ABSTRACT. The contribution of self-pollination to overall reproduction in *Isotria medeoloides* was experimentally assessed. We used insect exclosures and applied three treatments—no floral manipulation; pollinaria removal followed by hand self-pollination; and pollinaria removal followed by hand cross-pollination—and a control. Mature capsule dimensions and total seed weight were measured for each capsule. Analysis of variance revealed no significant treatment effects in mean seed weight, whereas a G-test showed that there were no differences in fruit set among treatments. Capsule length and diameter were strongly correlated with seed weight. Additionally, plant size (measured as leaf whorl diameter) was positively correlated with overall mean seed weight. We conclude that *I. medeoloides* is self-compatible and primarily self-pollinating. We also conclude that while reproduction is not limited by pollen availability, it may be limited by resources, assessed by overall plant size.

Key Words: *Isotria medeoloides,* Maine, autogamy, self compatibility

Isotria medeoloides (Pursh) Raf. has historically been considered the rarest orchid east of the Mississippi except in Florida (Luer 1975), and was included on the Federal List of endangered plant species in 1982 (Federal Register, 1982, vol. 47, p. 39827). However, this species was downlisted to Federally Threatened in 1994 (Federal Register, 1994, vol. 59, p. 50857). As of 1988, there were 81 known populations, the majority of which contain only a few (3–10) individuals (Gregory 1988; Mehrhoff 1983). *Isotria medeoloides* is a sympodial geophyte that produces a single (rarely 2) terminal flower.

The column of *Isotria medeoloides* has a terminal hinged anther which rests above the stigmatic area. The mealy pollen is extruded beyond the pollinia recesses hidden in the adaxial surface of the anther during the second day of anthesis. During pollen extrusion, the hinged anther is forced up, and the pollen is

then able to come into contact with the stigmatic area below (Mehrhoff 1980, 1983; pers. obs.). Although this floral morphology enables self-pollination, Mehrhoff (1980, 1983) pointed out that the small populations in his study area precluded experiments to determine the contribution of self-fertilization to overall reproduction. In contrast, the presence of several relatively large populations (greater than 100 individuals) in southern Maine allowed us to study the breeding system of this species as part of an ongoing demographic study.

There has been much anecdotal evidence regarding the breeding system of this species, as well as Mehrhoff's (1980, 1983) field observations that cite the lack of an observed insect vector. Despite the apparent lack of an insect pollinator, *Isotria medeoloides* has consistent, and sometimes quite high, levels of fruit set in natural populations (Vitt 1991). Specific knowledge of the reproductive biology of a rare species enhances our ability to interpret demographic trends, and may help us to predict the underlying genetic structure of a population. This study was designed to determine if *I. medeoloides* is self-compatible and to assess the relative contributions of self- and cross-pollination to overall reproduction.

MATERIALS AND METHODS

Our study site on a Nature Conservancy preserve in southern Maine is typical of the second growth mixed hardwood usually occupied by this species (Gregory 1988). Despite the large population size (200–300 plants), only 52 individuals were flowering when the study was initiated in early June of 1990. We defined an individual (genet) as a stem which occurred farther than 5 cm away from its nearest neighbor. This definition was based upon observations of the underground growth habits of a few individuals, and additionally upon the fact that the vast majority of emergent stems were sparsely dispersed as single stems over the study area. Detailed field observations were carried out from June 5 to June 20, 1989 and from June 1 to June 14, 1990. Approximately 40 hours between 0700 and 1300 hours, and 30 hours between 1300 and 1900 hours, were spent watching for insect visitors over the two field seasons. A preliminary experiment was done in 1989 to assess the occur-

rence of agamospermy in this species, while the primary experiment to test for autogamy was done in 1990.

The terminal anthers with intact pollinia (the pollinaria) of eight plants were carefully removed at the hinge with forceps in 1989 to test for agamospermy. We subsequently bagged four plants, and let the remainder open-pollinate. In 1990, we randomly assigned 40 flowering plants to one of three treatment groups: (1) no manipulation of the flower; (2) manual self-pollination subsequent to pollinaria removal; (3) manual cross-pollination with nearby individuals after pollinaria removal (Table 1). Pollinaria removal was carried out either before anthesis or during the morning of the first day of anthesis when there was no evidence of pollen extrusion from the pollinia recesses. After treatment, we covered the plants in all three groups with a fine mesh insect exclosure. The 12 plants in the control group were not covered.

Treatment 1 determined if levels of pollination would differ from the control when all insects were excluded (i.e., does autogamy occur in this species), while treatment 2 tested whether this species is self-compatible. Results from treatment 3 were compared with the control to determine if manual cross-pollination increased fruit set relative to open pollination. We expected to detect no differences among the treatments. Despite the lack of evidence of an insect pollinator, fruit set in natural populations can be as high as 89% (Vitt 1991). We reasoned that *Isotria medeoloides* would be highly self-compatible as well as strongly self-pollinating.

We considered pollination successful if the ovary had enlarged and appeared healthy two weeks after treatment. On August 26, 1990, we collected capsules and recorded maximum leaf-whorl diameter of each plant. Fruit set was recorded as negative when an individual initiated a fruit which was subsequently aborted.

We weighed the fresh capsules the day after collection. We used digital calipers and software provided by L. M. Marcus to measure capsule diameter and length. Orchid seeds are too small and numerous to count; therefore, we measured seed set as the total weight of the seeds in each capsule. Individual capsules were air dried in paper bags. The dried capsules remained intact during this process, and were weighed both before and after seed removal. We then calculated the percentage of all individuals that set fruit, as well as the mean total seed weight for each treatment.

RESULTS

In the 1989 preliminary study on agamospermy, 7 of the 8 plants failed to produce fruit. The only plant producing fruit was one that had been allowed to open-pollinate. Although the number of bagged plants from which the pollinaria had been removed in the preliminary study is small (only 4 individuals), the absence of fruit among these suggests agamospermy does not occur in this species. Although fruit set in the open-pollinated individual may have been the result of a cross-pollination event, it is also possible that pollination occurred prior to pollinaria removal.

No insect visitors were detected during our observations in either field season. In 1990, during an informal survey of all flowering individuals found at our study site, only four flowers were found with the pollinaria missing. We never observed pollinaria deposition.

Of the 42 individuals which initiated a capsule, two aborted, one from treatment 1 and one from treatment 2. These were not included in further analysis. Fruit set for the control group (no manipulation, no exclosure) was 83%, whereas fruit set for treatment 1 (no manipulation, exclosure) was 67% (Table 1). Both treatments 2 (hand self-pollination, exclosure) and 3 (hand cross-pollination, exclosure) resulted in 82% fruit set (Table 1), indicating that outcrossing does not increase fruit set, relative to selfing. A G-test of homogeneity between treatment 1 and the control revealed no significant differences ($G = 0.14$; $df = 1$; $0.5 < p < 0.9$), indicating that bagging alone does not significantly affect fruit set, relative to natural levels of pollination. In fact, a G-test for independence across all treatments found that fruit set was independent of treatment ($G = 0.224$; $df = 3$; $0.9 < p < 0.975$). Thus, insects are not necessary to produce fruit in this species, and autogamous pollination can account for observed levels of fruit set in natural populations.

Neither fresh capsule weight nor mean seed weight per capsule differed significantly between treatments using analyses of variance. Bagging (Treatment 1) had a marginally significant effect on capsule length ($F = 2.76$, $p = 0.0570$), compared to the control (Table 1). Correlations between capsule dimensions and overall seed weight were highly significant. Length of capsule was strongly correlated with seed weight (Spearman's $r = 0.86070$, $P < 0.0001$; $n = 37$), as was capsule diameter (Spearman's $r =$

Table 1. Summary of findings for experiments done in 1990. [a] Plants in treatments were covered with a mesh insect exclosure. [b] In grams; reported with Standard Errors. [c] In centimeters; reported with Standard Errors. * Means with different letters are significantly different from each other at the 0.057 level, segregated using Duncan's Multiple Range Test.

Treatment	n	Fruiting	Fruit Set	Capsule Length[c]*	Capsule Width[c]	Leaf Whorl Diameter[c]	Fresh Capsule Weight[b]	Mean Total Seed Weight[b]
(1) [a]No manipulation	18	12	67%	0.237 ± 0.009[f]	0.068 ± 0.006	13.99 ± 0.575	0.406 ± 0.067	0.011 ± 0.003
(2) [a]Hand self-pollination	11	9	82%	0.253 ± 0.009[fg]	0.078 ± 0.003	14.74 ± 0.565	0.502 ± 0.046	0.014 ± 0.002
(3) [a]Hand cross-pollination	11	9	82%	0.246 ± 0.009[fg]	0.071 ± 0.005	14.13 ± 0.493	0.411 ± 0.054	0.022 ± 0.011
Control	12	10	83%	0.279 ± 0.013[g]	0.082 ± 0.006	15.19 ± 0.669	0.589 ± 0.076	0.018 ± 0.003

0.89899, $P < 0.0001$; $n = 37$). There was also a positive correlation between plant size (measured as leaf whorl diameter) and overall mean seed weight (Spearman's $r = 0.63830$, $P < 0.0001$; $n = 37$). In other words, larger plants produced larger capsules that contained a greater mass of seeds.

DISCUSSION

The fantastic floral forms and complex pollination systems for which orchids are best known presumably have evolved to ensure outcrossing. However, autogamous terrestrial orchids are not uncommon, particularly in habitats that are harsh or where there is a paucity of pollen vectors (Dafni and Bernhardt 1990). The location of the anther above the stigma, coupled with friable pollen and the trend toward self-compatibility, is thought to be an adaptation for mechanical self-pollination (Burns-Balogh and Bernhardt 1985). This arrangement allows extrusion of the mealy pollen directly onto the stigma. *Isotria medeoloides* appears to follow the same morphological pattern (Mehrhoff 1983), and we have shown here that this species is self-compatible.

Although the mealy pollen may be available for outcrossing once it has been extruded from the pollinaria, a pollen vector has not been identified in this study. In the four cases where we assumed the pollinaria had been removed, it is possible that the pollen projection had broken and fallen to the labellum prior to contacting the stigmatic area. This phenomenon was reported by Mehrhoff (1980); pollination was not accomplished in such cases. A pollen vector was not found in the previous study conducted by Mehrhoff (1980), nor in many pieces of anecdotal evidence from others who are monitoring this species from Virginia to New Hampshire, nor was evidence of pollinaria deposition found in the current study. Mehrhoff (1980) concluded that autogamous pollination may have evolved in this species as a response to low pollinator availability, and, given the high levels of fruit set in this species, without apparent benefit of cross-pollination, we conclude that *Isotria medeoloides* reproduces primarily through autogamous self-fertilization. Although it appears that agamospermy does not occur in this species, we cannot rule out the possibility of pseudogamy.

Autogamous pollen deposition appears to be necessary to ensure fruit production. In fact, *Isotria medeoloides* may be thought

of as facultatively self-pollinating. However, only 67% of individuals in treatment 1 set fruit, compared with 83% in the control, and 82% in both treatments 2 and 3. Although this difference is not statistically significant, given our sample sizes, it is possible that an increased sample size would either corroborate our findings or might indicate that an as-yet-unobserved pollinator may be responsible for some of the natural levels of fruit set in this and other populations. Further study of the breeding system of this species may be warranted, at the least to determine if cross-pollination does occur as a rare event. This would lend a great deal of insight into the possible genetic structure of natural populations.

Orchid pollination systems are typically thought to be adaptations for low pollinator availability (Montalvo and Ackerman 1987; Ackerman 1989), but resource limitation also may explain the low levels of fruit set which are commonly found throughout the Orchidaceae. If manual pollination does not increase seed or fruit set relative to natural levels, reproduction then is thought to be limited by resources (Willson 1983). As we found no significant differences in capsule production or total seed weight between natural levels of pollination and hand self-pollination, it appears that reproduction in *Isotria medeoloides* is not pollen limited, but may be resource limited.

In fact, reproductive output in *Isotria medeoloides* is closely tied to plant size. The largest individuals produced the largest capsules and highest total seed weight. From our demographic work with *I. medeoloides* in Maine, we know that those individuals which produce fruit consistently belong in the highest size class (Vitt 1991). In addition, we have shown that *I. medeoloides* is self-compatible and primarily self-pollinating. The lack of increased fruit set under our experimental treatments when pollen is presumably not limiting versus the control when pollen may be limiting, indicates that reproduction is limited by resource availability. This conclusion is reinforced by the tight correlation between reproductive output and plant size.

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